An AMPK-stabilizing peptide ameliorates adipose tissue wasting in cancer cachexia in mice

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**SUMMARY**

Cachexia represents a fatal energy wasting syndrome in a large number of patients with cancer, mostly resulting in a pathological loss of skeletal muscle and adipose tissue. Here we show that tumor cell exposure of white adipocytes as well as tumor growth in mice triggered a futile energy wasting cycle in white adipose tissue (WAT). While uncoupling protein (Ucp)1-dependent thermogenesis was dispensable for tumor-induced body wasting, WAT from cachetic mice and tumor-cell supernatant treated adipocytes were consistently characterized by the simultaneous induction of both lipolytic and lipogenic pathways. Paradoxically, this was accompanied by an inactivated AMP-activated protein kinase (Ampk), which is normally activated in peripheral tissues during states of low cellular energy. Ampk inactivation correlated with its degradation and upregulation of Cidea. Thus, we developed an Ampk stabilizing peptide, ACIP, which was able to ameliorate WAT wasting *in vitro* and *in vivo*, mediated by shielding the Cidea-targeted interaction surface on Ampk. Thus, our data establish the Ucp1-independent remodeling of adipocyte lipid homeostasis as a key event in tumor-induced WAT wasting and propose the ACIP-dependent preservation of Ampk integrity in the WAT as a concept in future cachexia therapies.

**INTRODUCTION**

Cachexia represents a fatal energy wasting syndrome in up to 30% of tumor-bearing patients, most notably in cancers of the colon, the pancreas, and the lung[1](#_ENREF_1). Due to the phenotypic heterogeneity of cancer[2](#_ENREF_2), and the mostly unknown etiology at the molecular level, muscle and adipose tissue loss during cachexia still represents an immediate unmet medical need as effective and routine therapeutic measures to prevent or treat cancer cachexia are still lacking to date[3](#_ENREF_3). Interestingly, recent studies have challenged the classical view of cancer cachexia as a tumor-initiated event that eventually deprives host energy resources to feed tumor growth[4](#_ENREF_4) by highlighting the role of cachectic metabolism as an integrative, global response to tumor and/or host-derived stimuli that leads to the tissue wasting in this condition[5](#_ENREF_5),[6](#_ENREF_6).

While much attention has been focused on the muscle loss that occurs during cancer cachexia, it should be noted that depletion of white adipose tissue (WAT), which acts as the body’s main energy storage organ and fatty acid (FA) reservoir[7](#_ENREF_7), generally precedes the loss of skeletal muscle mass upon pathological tumor growth[8](#_ENREF_8). Also, biomarkers of WAT dysfunction have been proposed as prognostic and diagnostic markers in patients with cancer cachexia[9](#_ENREF_9). Both epidemiological and clinical studies have clearly demonstrated a beneficial, pro-survival effect of an above-normal body mass index during end-stage cancer diseases[10](#_ENREF_10), and the degree of WAT loss was found to serve as a survival predictor in weight-losing patients with pancreatic cancer[11](#_ENREF_11).

Indeed, cancer cachectic animals show a substantial substrate switch from carbohydrates to FAs towards the final stages of the disease as measured by a drop in the respiratory exchange ratio (RER)[12](#_ENREF_12),[13](#_ENREF_13). Because of the absence of this effect in pair-fed, non-tumor-carrying animals, this substrate switch cannot be explained by simple starvation but rather points to tumor-specific alterations in systemic lipid homeostasis during the advanced stages of cancer cachexia.

Two recent studies have described a white-to-brown adipocyte switch, *i.e.* WAT “browning”, as a component of WAT dysfunction in cancer cachexia[12](#_ENREF_12),[13](#_ENREF_13), arguing for a role of uncoupling protein (Ucp)1-dependent thermogenesis in cancer cachectic body wasting in certain tumor entities. As part of the “browning” program, cell death-inducing DFF45-like effector (Cide) a, a member of the lipid droplet-associated Cide family, has emerged as important metabolic regulator[14](#_ENREF_14). Indeed, *Cidea*-null animals are characterized by elevated energy expenditure[15](#_ENREF_15), and CIDEA levels have been shown to be increased in the WAT of patients with cachexia[16](#_ENREF_16). However, a genetic proof of the importance of Ucp1 and other “browning” markers in the cachexia context has not been obtained thus far, and the majority of human studies has failed to demonstrate a significant increase in resting energy expenditure in the cancer cachectic state[17](#_ENREF_17), overall arguing for the existence of alternative, as-yet non-defined mechanisms in tumor-driven WAT remodeling and dysfunction.

AMPK is an evolutionarily conserved, heterotrimeric serine/threonine protein kinase. Upon a drop in cellular energy levels, AMPK is activated through an allosteric conformational change in its gamma subunit and a subsequent activating phosphorylation of its alpha subunit, ultimately turning on ATP-generating catabolic processes[18](#_ENREF_18). *Ampk* beta1 KO mice display reduced adiposity and body fat content and tend to have elevated levels of circulating fatty acids, indicative of enhanced lipid breakdown[19](#_ENREF_19). Also, previous studies have demonstrated an anti-lipolytic effect of chronic Ampk activation in adipose tissue stores in cellular and animal models[20-22](#_ENREF_20), as well as in patients with type 2 diabetes[23](#_ENREF_23). Furthermore, anti-diabetic drugs of the biguanide and thiazolidinedione families have been shown to inhibit lipolysis in an AMPK-dependent manner in human adipocytes[24](#_ENREF_24), overall supporting the concept that under certain conditions AMPK inhibition can promote lipid mobilization. Here we identify the lack of Ampk activity as a common feature of adipose tissue dysfunction in cancer cachexia in mice, triggered –at least in part- through the aberrant induction of Cidea and the subsequent degradation of Ampk in this tissue. In addition, we show that treatment of cachectic animals with a peptide specifically targeting the WAT Ampk-Cidea interaction prevents adipose tissue loss under cachectic conditions.

**RESULTS**

***WAT remodeling during cancer cachexia is characterized by Ampk inactivation***

Metabolic dysfunction in WAT has not yet been considered in the clinical definition of cancer cachexia[25](#_ENREF_25), prompting us to initially define the cachectic response of adipocytes upon direct exposure to tumor-secreted factors. To this end, we treated mature 3T3-L1 adipocytes with tumor cell-conditioned medium (CM) from murine (C26, MC38) and human (SW480, HT29) colon as well as lung cancer (LLC) cells in a cell autonomous experimental setup. CM of tumor cell lines causing cachexia *in vivo*, including C26[26](#_ENREF_26), LLC[27](#_ENREF_27), and SW480[28](#_ENREF_28) led to higher adipocyte lipolysis as compared to non-cell conditioned control medium, while CM of the non-cachexia-inducing colon cancer cell lines MC38[29](#_ENREF_29) and HT29[28](#_ENREF_28) did not induce release of non-esterified fatty acids (NEFAs) (**Fig. 1a**), in line with recent studies demonstrating that WAT lipolysis represents a key factor behind cancer cachexia in weight-losing human patients[30](#_ENREF_30). In addition, exposure of primary mouse adipocytes to serum from cachectic C26 tumor-carrying mice resulted in a higher release of NEFAs compared to primary adipocytes treated with serum of non-cachectic control mice (**Fig. 1b**). Consistent with these findings, adipocyte triglyceride (TG) content in 3T3-L1 adipocytes was lower in response to cachexia-inducing CM as compared with non-cell conditioned control medium (**Fig. 1c**). Intriguingly, compared to control CM, tumor cell CM from C26, LLC, and SW480 cells also resulted in greater lipogenesis in mature 3T3-L1, and serum from C26 tumor-carrying mice induced lipogenesis in primary mouse adipocytes when compared to serum from non-cachectic animals (**Fig. 1d**,**e**). In addition, mature 3T3-L1 adipocytes treated with C26 CM displayed significantly lower maximal respiratory capacity than control CM treated 3T3-L1 adipocytes (**Fig. 1f**). These results point towards an energy costly lipid cycling induced by tumor-borne pro-cachectic signals. In congruence with this notion, ATP levels were significantly lower in 3T3-L1 adipocytes treated with C26 CM when compared to control CM or CM of the non-cachexia inducing cell line MC38 (**Fig. 1g**). To next define the molecular events leading to cachectic adipocyte metabolism, we analyzed the protein expression of key enzymes involved in lipolytic and lipogenic pathways. While we did not observe a higher expression or activating phosphorylation of hormone sensitive lipase (Hsl) (data not shown), which could explain the greater adipocyte lipolysis observed in cells treated with conditioned media from cachetic cell lines, Western blot analysis demonstrated that the inhibitory Hsl phosphorylation at S565[31](#_ENREF_31) was blunted in adipocytes treated with C26 or LLC CM (**Fig. 1h**), suggesting that tumor cell-derived pro-cachectic factors abolish a major brake in lipid mobilization in adipocytes, thereby promoting loss of energy stores under these conditions. In addition, the inhibitory phosphorylation of the key lipogenic enzyme acetyl-CoA carboxylase (Acc)[32](#_ENREF_32) was lower in cachectic CM treated cells compared to non-cell conditioned control mediumtreated cells, underlining the simultaneous induction of both lipolysis and lipogenesis as demonstrated above.

Both Hsl-S565 and Acc-S79 phosphorylation have previously been identified as substrates for Ampk[31](#_ENREF_31),[33](#_ENREF_33),[34](#_ENREF_34). While Ampk activity is typically increased in response to energy deprivation[18](#_ENREF_18), and we indeed noticed lower cellular ATP levels in the “cachectic” adipocytes compared to control CM-treated cells (**Fig. 1f**), further analysis documented a significantly lower degree of protein expression of both Ampk subunits alpha and beta in adipocytes exposed to cachexia-inducing CM as compared with cells treated with control CM (**Fig. 1h**), suggesting that in adipocytes treated with supernatants from cancer cachectic tumor cell lines there is a paradoxical disruption of the functional integrity of Ampk despite low cellular energy levels.

To test whether the inactivation of Ampk represents a critical pathophysiological event in WAT dysfunction during cancer cachexia and verify the functional significance of the adipocyte-autonomous effects *in vivo*, we next investigated WAT Ampk activity in a range of mouse models of cancer cachexia. Consistent with our previous study[35](#_ENREF_35), C26 tumor cell implantation caused a massive reduction in body weight, skeletal muscle and overall fat mass, as well as depot-specific inguinal (iWAT), abdominal (aWAT) and brown (BAT) adipose tissue mass after 21 d (**Supplementary Fig. 1a**,**b**). Histological examination of WAT depots revealed smaller lipid droplets in WAT of cachectic animals compared to non-tumor bearing mice, irrespective of whether they were pair-fed to cachectic animals or random fed (**Supplementary Fig. 1c**). This was consistent with higher levels of circulating NEFA in tumor-bearing animals (**Supplementary Fig. 1d**), indicative of enhanced lipolytic activity as previously observed in patients[8](#_ENREF_8).

Mimicking the paradoxical condition in tumor cell CM-treated adipocytes, Western blot analysis of WAT of C26 cachectic animals showed lower levels of Hsl-S565 and Acc-S79 phosphorylation as well as lower protein expression of total and phosphorylated Ampk alpha and beta (**Fig. 2a**,**b**). In addition, ATP levels were lower in the WAT from cachectic mice while two key markers of lipogenic and glyceroneogenic gene expression were higher compared to non-tumor-bearing control animals (**Fig. 2c**,**d**), thereby reflecting the simultaneous induction of lipolytic and lipogenic/re-esterification pathways as observed *in vitro* (**Fig. 1**). Indeed, Ampk beta could hardly be detected in immunoprecipitates of Ampk alpha from the WAT and BAT of cachectic animals, while Ampk beta was readily recovered in healthy control WAT and BAT depots (**Fig. 2e**). Consequently, Ampk enzymatic activity was substantially lower in WAT and BAT from cachectic mice as compared with healthy controls in *ex vivo* substrate utilization assays, reflecting a change in the activities of both Ampk alpha1 and alpha2 isoforms (**Fig. 2f**).

Of note, tumor-bearing mice started to lose weight two weeks after tumor cell injection, the effect of which became more severe towards the three week time point (**Supplementary Fig. 1e**). The initial loss in adipose tissue mass corresponded with an elevation of Cidea and a minor loss in Ampk alpha and an almost significant reduction in Ampk beta. These effects then became significant after more prolonged tumor exposure after three weeks (**Supplementary Fig. 1f**-**h**). Thus, the elevation of Cidea and the reduction in Ampk beta levels occurred at rather early time points during cachexia development.

To functionally verify the role of Ampk in the observed phenotypes, we next employed genetic re-constitution assays in isolated adipocyte systems. Serum from cachectic animals triggered lipolysis in cultured adipocytes as shown above. Re-constitution of a constitutively active Ampk alpha protein partially prevented this lipolytic response (**Fig. 2g**), demonstrating that Ampk inhibition mediates significant parts of the lipolytic phenotype upon exposure to pro-cachectic stimuli. Of note, none of the observed changes in Ampk expression and signaling were observed in WAT depots from mice transplanted with MC38 colon cancer cells that do not cause any cachectic phenotypes *in vivo* (**Supplementary Fig. 1i**)[6](#_ENREF_6) and *in vitro* (**Fig. 1**).

While the role of increased adipocyte triglyceride lipase (Atgl) and Hsl activity in cachectic WAT is well established[36](#_ENREF_36), we did not observe a strong increase in lipase gene expression or classical beta-sympathetic innervation of WAT as demonstrated by Western blot showing Hsl-S660 and protein kinase A target phosphorylation (**Supplementary Fig. 1j**) and by chemical blockade of sympathetic nerve endings of tumor-bearing and control littermates. Depot-specific injection of 6-OHDA (6-hydroxydopamine) into inguinal fat pads, which effectively blocks adrenergic signaling[37](#_ENREF_37), had no influence on WAT wasting and remodeling in cachectic animals (**Supplementary Fig. 2a**-**c**). In contrast, 6-OHDA injection significantly reduced norepinephrine levels specifically in iWAT and blunted cold-induced white-to-brown adipocyte conversion in iWAT depots (**Supplementary Fig. 2d**,**e**), demonstrating that the compound *per se* works as anticipated. In congruence with these findings, inhibition of cAMP signaling in cultured adipocytes by H89 inhibitor treatment did not impair the CM-triggered lipolytic response (**Supplementary Fig. 2f**). Thus, our data confirm the importance of increased lipolytic and lipogenic activity in cachexia, mainly by loss of internal breaks on lipolysis.

To further substantiate these results in a genetic model of colon cancer-induced wasting metabolism, we employed animals carrying an *Apc* mutation (APC580/+), which develop numerous intestinal lesions at five to six months of age[38](#_ENREF_38). Lesion appearance triggered a significant reduction in body weight, skeletal muscle and WAT mass in relation to healthy controls (**Supplementary Fig. 3a**,**b**). Importantly, *Apc* mutant cachectic animals also displayed elevated NEFA levels in serum (**Supplementary Fig. 3c**) as well as decreased expression and activity of Ampk in WAT (**Fig. 2h**).

In order to extend the relevance of our findings into a humanized setting, we next implanted immunocompromised Fox Chase SCID and nude BALB/c mice with human SW480 colon cancer cells[28](#_ENREF_28). SW480 tumors after 14 d induced rapid body weight loss, reduced WAT and muscle mass, and elevated expression of the ubiquitin ligase genes *Atrogin-1* and *Murf1* as well as autophagy marker *Bnip3* in skeletal muscle (**Supplementary Fig. 3d**-**f**). In line with the effects of the mouse tumors, human colon cancer cells induced a decrease in Ampk alpha and beta expression and activation as assessed by Hsl and Acc phosphorylation (**Fig. 2i** and **Supplementary Fig. 3g**), overall promoting the notion that colon cancer-associated cachexia is characterized by WAT Ampk inactivation and the subsequent promotion of energy-costly lipid turnover due to parallel increases in lipolysis, lipogenesis, and re-esterification.

***Energy wasting in cancer cachexia is not dependent on Ucp1***

Given the recent findings describing WAT “browning” as a component of WAT dysfunction in cancer cachexia and their suggestion for a role of Ucp1-dependent thermogenesis in cachexia caused by certain tumor entities[12](#_ENREF_12),[13](#_ENREF_13), we explored the relevance of these findings under our experimental conditions. While we observed the reported induction of a distinct component of the brown adipocyte thermogenic program in WAT upon tumor growth, *i.e.* the elevation of Cidea mRNA and protein levels (**Fig. 2a**,**h**-**i** and **Supplementary Fig. 4a**), no specific Ucp1 staining was observed in the iWAT of either the C26 or LLC mouse model. The iWAT of tumor-induced *Ucp1* KO mice and the iWAT of cold-exposed wild-type animals served as negative and positive controls, respectively (**Fig. 3a**).

C26 tumor cell implantation caused a sharp decrease in body weight over the last days before death (**Supplementary Fig. 4b**), which was associated with a significant decrease in resting metabolic rate, average oxygen consumption and CO2 production (**Supplementary Fig. 4c**-**e**),again in line with a minor impact of Ucp1-mediated thermogenesis. Indeed, when calculated according to the consensus ANCOVA statistical procedure[39](#_ENREF_39), resting metabolic rate of end-stage cachectic animals remained below the rate in healthy, pair-fed controls (**Supplementary Fig. 4f**). Of note, while food intake shortly peaked before the final disease stages (**Supplementary Fig. 4g**), physical activity remained largely unaffected (**Supplementary Fig. 4h**), and the RER was significantly decreased as compared with pair-fed littermates with no tumors (**Supplementary Fig. 4i**), underlining a shift towards fatty acid catabolism during the final disease stages. Also, none of the additional classical brown adipocyte markers, including *Dio2*, *Cox7a1* and *Prdm16*, were higher in cachectic compared to control WAT (**Supplementary Fig. 5a**,**b**).

To further test for the potential contribution of thermally-induced WAT browning and/or BAT activity to the cancer cachexia phenotype in the C26 model, we next placed the animals at a thermoneutral 30 °C housing temperature, which effectively abrogates sympathetic BAT activation by thermal stimuli[40](#_ENREF_40). Importantly, housing of experimental animals at thermoneutral conditions throughout the tumor growth period did not alter the observed WAT remodeling nor did it abrogate the loss of WAT upon tumor growth and the elevation of circulating NEFA levels (**Fig. 3b-e**), again arguing for wasting cycles in the tumor bearing state that were independent of classical sympathetic BAT activation.

To ultimately test this hypothesis at a genetic level, we employed *Ucp1* knockout mice[41](#_ENREF_41) in syngeneic transplantation experiments using LLC tumor cells which induced mild cachexia as indicated by body weight, body fat, WAT and skeletal muscle loss (**Supplementary Fig. 5c**) thereby mimicking the observed cachectic features in the C26 transplantation model. As observed in the C26-injected animals, the WAT of LLC-induced cachectic mice showed lower expression of Ampk alpha and beta and higher expression of Cidea compared to WAT of non-tumor injected littermates (**Supplementary Fig. 5d**). Consistent with the absence of significant Ucp1 induction and no overt increase in oxygen consumption and resting metabolic rate also in C26 tumor-bearing animals (**Supplementary Fig. 5a** and **Supplementary Fig. 4c** ), *Ucp1* knockout mice still displayed body weight loss and adipose tissue as well as muscle mass wasting identical to their wild-type littermates (**Fig. 3f**-**i**) and loss of *Ucp1* did not significantly alter systemic oxygen consumption rates under cancer cachectic conditions (**Supplementary Fig. 5e**), indicating that the impact of Ucp1-mediated thermogenesis on cancer cachectic phenotypes may be less pronounced than previously anticipated. In this regard, our results thus far argue for the existence of a Ucp1-independent WAT remodeling upon cancer cachectic conditions characterized by the loss of Ampk signaling, the induction of only a subset of brown adipocyte marker genes, for example, *Cidea*, and the simultaneous activation of lipolytic and lipogenic pathways.

***Cachexia-triggered Ampk degradation is partly mediated by Cidea***

To next explore the mechanism leading to decreased Ampk levels and activity in cachectic WAT, we analyzed Ampk gene expression in more detail and found that WAT *Ampk* alpha and beta mRNA levels were in fact unchanged in cachexia (**Fig. 4a**), suggesting that the regulation of Ampk may rather occur at the protein level. Indeed, Ampk beta protein stability decreased in 3T3-L1 adipocytes treated with C26 cell supernatant (**Supplementary Fig. 6a**).

Intriguingly, Cidea as a component of the above demonstrated WAT remodeling process during cachexia has been described to interact with Ampk, leading to destabilization of the Ampk complex and proteasomal degradation of the kinase[42](#_ENREF_42). While Cidea is generally considered specific to BAT in rodents[43](#_ENREF_43), we observed increased *Cidea* mRNA expression in both white and brown fat from cachectic mice as shown above and also by others (**Supplementary Fig. 4a)**[12](#_ENREF_12),[13](#_ENREF_13). Furthermore, Cidea protein was overexpressed in the WAT from cachectic mice (**Fig. 2a**,**h**,**i**), and siRNA-mediated *Cidea* knockdown (**Supplementary Fig. 6b**) blunted C26 CM-triggered lipolysis in adipocytes (**Fig. 4b**), supporting the hypothesis that the Cidea-Ampk beta interaction may represent a specific molecular target in tumor-induced WAT remodeling and energy loss. Due to their overall lean phenotype[15](#_ENREF_15), LLC tumor-carrying *Cidea* KO mice did not differ in their final body and adipose tissue weights from wild-type littermates as anticipated (**Supplementary Fig. 6c**). However, LLC tumor cell-implanted *Cidea* KO animals harbored higher levels of phosphorylated Hsl-S565 and Ampk beta protein in WAT as compared with wild-type LLC injected littermates (**Fig. 4c**). In addition, exposure of WAT explants from wild-type and *Cidea* KO mice to serum from cachectic C26-bearing mice triggered lipolysis in wild-type but not *Cidea*-deficient adipose tissue (**Fig. 4d** and **Supplementary Fig. 6d**), further demonstrating the necessity of Cidea for the tumor-induced lipolysis and degradation of Ampk in WAT at a genetic level.

Indeed, in WAT biopsies from 27 patients with varying degrees of cancer cachexia, *CIDEA* levels significantly correlated with the degree of weight loss in these subjects (**Fig. 5a**,**b**), thereby supporting the notion that the described pro-cachectic mechanism also applies to the human situation. Of note, the human cohort included herein was very unique as no confounding chemotherapy was involved, but still rather small as it was difficult to obtain sufficient WAT from adipose tissue-losing individuals. Thus, although a larger sample size would have been necessary to further increase statistical power, this was not possible within this particular patient cohort. Also, due to technical limitations an analysis of AMPK protein levels was not feasible with the human material available. However, in order to still further corroborate these findings, we analyzed a second, completely distinct cohort of more than 60 cachectic and non-cachectic tumor-carrying subjects. *CIDEA* mRNA levels were increased in both subcutaneous and abdominal WAT depots from cachectic individuals as compared with non-cachectic controls also in this cohort (**Fig. 5c**), further supporting the notion that WAT *CIDEA* is positively associated with cachectic conditions in humans. Intriguingly, the analysis of 23 heavily obese subjects who underwent bariatric surgery showed that the induction of *CIDEA* levels in omental WAT during the first 12 month after the surgery (**Fig. 5d**) significantly correlated with an increase in circulating NEFA levels (**Fig. 5e**), suggesting that the elevation of *CIDEA* in human WAT and the induction of lipolysis are tightly coupled under conditions of substantial weight loss in humans.

Based on these results we then tested for the importance of Ampk at the functional level. We designed a peptide coding for Ampk beta1 amino acids 232-248, shielding the Cidea-Ampk beta interaction surface to block inter-molecular interaction at this site (thus we named it, ACIP for Ampk-Cidea Interfering Peptide), and thereby preventing Ampk degradation. The designed ACIP peptide was indeed able to efficiently bind to Cidea in cellular transfection assays (**Fig. 5f**). Importantly, co-transfection of ACIP with a tagged version of Cidea impaired the Cidea-Ampk beta interaction as demonstrated by co-immunoprecipiation assays using mouse embryonic fibroblasts (**Fig. 5g**). Transfection of 3T3-L1 cells with ACIP prevented the Ampk de-stabilization by C26 cell supernatant (**Supplementary Fig. 6a**). In line with the co-immunoprecipitation data from cachectic animals (**Fig. 2e**), exposure of primary white adipocytes to serum from cancer cachectic, C26 tumor-carrying mice abolished the interaction between the Ampk alpha and beta subunits (**Fig. 5h**). Delivery of the inhibitory ACIP peptide into these cells partially restored the Ampk alpha-beta interaction (**Fig. 5h**), thereby leading to the reconstitution of Ampk substrate phosphorylation, most notably Hsl-S565 and Acc-S79 (**Fig. 5i**). In congruence with the molecular rescue of Ampk activity, treatment of adipocytes with the Ampk activator AICAR inhibited the C26-mediated stimulation of lipolysis (**Supplementary Fig. 6e**), and ACIP delivery prevented NEFA and glycerol release from C26 mouse serum-treated primary white adipocytes (**Fig. 5j**). Overall, these results demonstrated that the ACIP peptide was able to counteract tumor-induced lipid breakdown in adipocytes by preserving Ampk anti-lipolytic and -lipogenic activity.

***Protection of Ampk stability ameliorates WAT dysfunction in cancer cachexia***

To explore the possibility that ACIP may represent a therapeutic approach to counteract tumor-induced WAT depletion by preventing Cidea-Ampk beta interaction, we generated an adeno-associated virus (AAV) carrying ACIP (**Fig. 6a**). Peptide expression was driven by the adipose tissue-specific *adiponectin* promoter, and limited to adipose tissue by the liver-specific miRNA122 binding site. ACIP-carrying and empty control AAVs were injected by microinjection directly into the right and left iWAT depots, respectively, and mice were rendered cachectic by C26 tumor cell implantation. Histological and expression analysis demonstrated that ACIP mRNA and peptide could specifically be detected exclusively in the injected iWAT depots but not in the control-injected iWAT nor in non-injected aWAT and liver from the same animal (**Supplementary Fig. 7a**,**b**). Consistent with data from isolated adipocytes, ACIP expression led to an average 30% greater iWAT depot weight under tumor-bearing conditions, correlating with larger adipocyte lipid droplet sizes compared to control-injected fat depots as demonstrated by histological examination (**Fig. 6b**,**c**). In contrast, ACIP had no major effect in iWAT depots from healthy control animals (**Fig. 6b**). In congruence with an Ampk and thus lipid preserving effect, local ACIP delivery rescued the inhibitory Hsl-S565 and Acc-S79 phosphorylation in cachectic animals, and resulted in greater Ampk alpha and beta levels as compared with control-treated, tumor-bearing animals (**Fig. 6d** and **Supplementary Fig. 7c**), demonstrating that the inhibitory ACIP peptide efficiently counteracts the major cachexia-driven WAT phenotypes upon local administration *in vivo*.

To finally explore the potency of the ACIP peptide at the systemic level and circumvent the depot restrictions of the site-directed injection approach, we employed the ACIP AAV vector system via systemic delivery. We injected mice with the ACIP- and control AAVs by tail vein injection and after a 15 d recovery period, C26 tumor cells were implanted. 21 d after tumor cell implantation, control AAV-injected animals displayed all signs of cancer cachexia, including a substantial loss of body weight, body fat, lean mass and WAT (**Fig. 6e**,**f**). Remarkably, injection of ACIP-carrying AAV led to an adipose tissue-specific ACIP expression profile (**Supplementary Fig. 7d**) and significantly reduced main signs of tumor-induced cachexia in WAT depots (**Fig. 6e**,**f**), most notably including a significant amelioration of iWAT and aWAT loss upon tumor growth while leaving the actual tumor size unaffected (**Fig. 6f** and **Supplementary Fig. 7e**). In non-tumor-bearing mice, ACIP did not significantly influence body weight or body composition (**Fig. 6e**,**f**). Consistent with these physiological effects, ACIP delivery restored the Ampk alpha-beta interaction in WAT of cancer cachectic animals (**Fig. 6g**), and rescued phosphorylation of Hsl-S565 and Acc-S79 in this tissue (**Fig. 6h**). Importantly, while we had to sacrifice almost all control-injected animals for ethical reasons due to a substantial 10-15% weight loss after 22 d of tumor growth, roughly 50% of ACIP-treated mice had not reached this degree of cachexia at this time point (**Fig. 6i**).

To ultimately confirm these findings in an independent, humanized setting, we employed human SW480 colon cancer cells in immunocompromised mice transplantation models. Tumor cell engraftment in these animals significantly reduced body weight and WAT stores, correlating with reduced Ampk levels as shown above (**Supplementary Fig. 3d**,**e**,**g,** and Fig. **2i**). Consistent with our findings in the murine C26 setting, systemic ACIP delivery promoted significant gains in both body and WAT depot weights (**Fig. 6j**), demonstrating that the ACIP-mediated restoration of Ampk protein levels and activity in WAT has the potential to enhance the well-being of tumor-bearing animals and to efficiently counteract WAT wasting under cancer cachectic conditions.

**DISCUSSION**

Cancer cachexia significantly contributes to high mortality rates, poor quality of life and resistance to chemotherapy[44](#_ENREF_44). Clinical attempts to ameliorate the cachectic phenotype by nutritional supplementation of energy-dense diets have proven to be largely ineffective in counteracting cancer cachexia in patients[45](#_ENREF_45). These data are in line with pair-feeding animal studies, demonstrating that cancer cachexia triggers systemic energy wasting beyond a sole reduction in food intake (*i.e.*, anorexia)[5](#_ENREF_5). In addition, also anti-inflammatory therapeutic regimens, including neutralizing antibodies against tumor necrosis factor alpha[46](#_ENREF_46), have provided only limited clinical success thus far, underscoring the complexity of pro-cachectic signaling events and thus limiting the potential of circulating, tumor-and host-borne factors to serve as effective therapeutic targets in cancer cachexia. Indeed, in line with this assumption, previous studies have all reached the conclusion that cancer cachexia is driven by the combinatorial action of multiple secreted macromolecules[6](#_ENREF_6),[12](#_ENREF_12),[13](#_ENREF_13).

The beneficial effects of ACIP demonstrated in this study suggest that the efficient and specific reconstitution of Ampk activity in the WAT by peptide delivery paradigms may provide a broad energetic, and thus survival, benefit in the tumor-bearing situation and serve as platform technology for novel anti-cachectic therapies. While Ampk alpha expression seems to change in both directions as part of a disrupted circadian gene expression rhythm during cancer cachexia[47](#_ENREF_47), our combined results from five independent models suggest that Ampk protein levels show an overall decrease during the final stages of cancer cachexia in mice, supported by the beneficial effects of ACIP treatment on WAT integrity through Ampk stabilization in the cancer-bearing state. Of note, as expected by their overall lean phenotype[15](#_ENREF_15), whole-body *Cidea* knockout mice were not generally protected against systemic cachexia phenotypes upon tumor cell implantation, further underlining the necessity to develop tissue-specific interference measures as components of an overall combinatorial cancer cachexia therapy, simultaneously directed against a number of dysfunctional parameters[48](#_ENREF_48). In this respect, ACIP may provide a necessary degree of specificity as general inhibition of Cidea could be anticipated to cause worsening rather than amelioration of cancer cachexia phenotypes in terms of body wasting and energy preservation. Indeed, *Cidea*-null animals are characterized by elevated energy expenditure[15](#_ENREF_15), which could neither be observed in cancer cachectic animals nor in most of the clinical studies investigating patients with cancer cachexia[17](#_ENREF_17). Interestingly, the induction of Cidea expression in WAT under cachectic conditions that we observed in the present study seems to be part of a partial, “brown-like” WAT remodeling process that has also been observed in humans[16](#_ENREF_16). While also the induction of the prototypical brown adipocyte marker gene Ucp1 has recently been correlated with tumor-induced body wasting[12](#_ENREF_12),[13](#_ENREF_13), the overall activation of BAT and/or brown adipose tissue markers, including Ucp1 expression, in murine and human cachexia seems variable, and the functional contribution of BAT activity to tumor-induced wasting had been uncertain thus far[48](#_ENREF_48). Our combined data from thermoneutrality, chemical denervation and genetic *Ucp1* knockout models now clearly argue against Ucp1-dependent thermogenesis as a common and stand-alone driving force in cancer cachectic body wasting. It is tempting to speculate that Ucp1-mediated thermogenesis may or may not modulate the degree of cachectic phenotypes that are mainly driven by a WAT remodeling process and Ampk dysfunction, depending on the actual cancer stage and tumor entity. The latter parameters (stage/entity) might also -at least in part- explain the discrepancies observed between our study and previously published results highlighting WAT “browning” as a determinant of cancer cachexia[12](#_ENREF_12),[13](#_ENREF_13). However, in contrast to our ANCOVA-based evaluation of oxygen consumption rates in cachectic animals, which actually showed a decrease during the course of the experiment, previous studies[13](#_ENREF_13) obtained their elevated rates mainly through the normalization of the raw data by reduced body weights, which might explain parts of the discrepancies between the two studies. In addition, the full contribution of WAT browning to overall energy expenditure as compared to classical BAT is still under debate. Despite the mild induction of *Ucp1* mRNA levels in tumor-exposed WAT in previous studies[12](#_ENREF_12),[13](#_ENREF_13), the absolute levels of WAT Ucp1 mRNA and protein still remain substantially lower as compared to BAT, thereby questioning the overall impact of WAT Ucp1-dependent thermogenesis to systemic energy homeostasis. In fact, along with our current data other investigators have observed the independence of cachectic phenotypes from classical BAT activation pathways as demonstrated by tumor implantation experiments under thermoneutral conditions[12](#_ENREF_12).

While lipolysis does not represent an energy costly process *per se*, even during fasting as much as 30-40% of NEFA liberated by the breakdown of stored TG are re-esterified to TG. This cycling creates a highly energy-demanding “futile” process, characterized by substantial ATP depletion and AMP elevation, ultimately leading to Ampk-mediated feedback inhibition of lipolysis under healthy conditions[49](#_ENREF_49). *In vitro* studies demonstrated that the rate of NEFA re-cycling proportionally increases with accelerated lipolytic flux[50](#_ENREF_50), and human patients with cachexia are found to display enhanced NEFA-TG cycling activity in WAT as determined by metabolic labeling studies[51](#_ENREF_51). Interestingly, Cidea seems to be especially important for tumor cell-stimulated lipolysis rather than basal lipolytic responses. Of note, while the mechanistic basis for this effect remains unclear, in particular “stimulated” lipolysis is aberrantly elevated in cancer cachectic conditions[52](#_ENREF_52) which may further underline the importance of the Cidea-Ampk axis in this context.

In line with our data and previous reports demonstrating ameliorated cachexia in *Atgl* and *Hsl* knockout animals[36](#_ENREF_36) and the induction of lipase activity in other cancer cachexia models[12](#_ENREF_12), early “adipocentric” treatment and restoration of WAT functional integrity may thus represent a critical therapeutic sub-component to slow down or prevent systemic body and skeletal muscle wasting[8](#_ENREF_8). This holds particularly true for the fact that WAT wasting seems to occur both early in the pathogenesis and independent from loss of skeletal muscle mass (this study and refs. 12,13). Intriguingly, systemic adipose tissue wasting in hyperhomocysteinemia, as a main risk factor for coronary heart disease[53](#_ENREF_53) and demonstrated to be correlated with progressive tumor-induced body weight loss[54](#_ENREF_54), as well as septic burn injury[55](#_ENREF_55) can be traced back to a loss in Ampk-dependent inhibition of WAT lipolysis. In fact, conditions of septic burn injury trigger the loss of Ampk alpha protein expression, correlating with reduced Hsl-S565 phosphorylation but no alterations in the Hsl activating modification at S660[55](#_ENREF_55), thereby mimicking the cancer cachectic conditions as described in this study and suggesting a broader importance of the Ampk-dependent anti-lipolytic pathway in critically ill subjects. Further in-depth human studies are required at this point to explore the contribution of WAT wasting to the overall cachectic phenotype. This represents a particular challenge because data variability in humans with cancer cachexia is high as weight loss is a self-reported parameter and it is often difficult to obtain exact information on spontaneous changes in body weight over time among free-living subjects.

In summary, our data imply that a molecular-metabolic dysfunction of WAT should be considered as an extension of the current clinical definition of cancer cachexia, while the restoration of WAT integrity deserves increased attention in future therapeutic approaches to target tumor-induced body wasting.

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**AUTHOR CONTRIBUTIONS**

M.R., M.S., V.L., B.E.Ü., K.N., C.A., O.H., T.P.S., A.Z., D.M., N.P., M.R., A.K., I.D., P.A., B.C., E-Z.A., B.K., G.R.S., P.J., J.K., C.W., M.B., and M.B.D. performed experiments and generated experimental tools. S.H. designed and directed research and wrote the manuscript.

**The authors declare no competing financial interest.**

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**FIGURE LEGENDS**

**Figure 1** Tumor cell exposure induces lipolysis, lipogenesis and energy crisis in adipocytes.(**a**)Non-esterified fatty acid (NEFA) levels in supernatants of 3T3-L1 adipocytes treated with C26, MC38, LLC, SW480 and HT29 cell-conditioned media (CM) (*n* = 6). (**b**) NEFA levels in supernatants of mouse primary adipocytes treated with control or C26 mouse serum (*n* = 4). (**c**)Cellular triglyceride (TG) content of 3T3-L1 adipocytes treated with C26 CM (*n* = 6). TG levels normalized to cellular protein content. (**d**) Lipogenesis in 3T3-L1 adipocytes upon treatment with C26, MC38, LLC, SW480 and HT29 CM (*n* = 6). (**e**) Lipogenesis in mouse primary adipocytes upon treatment with control or C26 mouse serum (*n* = 4). (**f**) Oxygen consumption rate of 3T3-L1 adipocytes treated with C26 CM. Oligomycin, FCCP and Antimycin A together with Rotenone were injected at the indicated time points. Data normalized to cell density (*n* = 29). (**g**) Cellular ATP content of 3T3-L1 adipocytes treated with C26, MC38 and LLC CM (*n* = 4). (**h**) Immunoblots (*n* = 3 per condition)of the lipolysis, lipogenesis and Ampk signaling pathways in 3T3-L1 adipocytes treated with C26, MC38 and LLC CM. Quantitation of pHsl-Ser565 over total Hsl (left) and pAcc-Ser79 over total Acc (right) protein levels (*n* = 3). All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates. (**a,d,f**) 1-way ANOVA with Tukey’s Multiple Comparison Posttest. (**b,c,e**)Student’s t-test. (**g**) 2-way ANOVA with Bonferroni’s Multiple Comparison Posttest. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Figure 2** Diminished Ampk activity in iWAT is a key feature of various cancer cachexia mouse models.(**a**) Immunoblot (representative for *n* = 5 per condition) of the lipolysis, lipogenesis and Ampk signaling pathways in iWAT of C26 cachectic animals. (**b**) Quantification of Hsl-S565 levels normalized to total Hsl protein levels as shown in a (*n* = 3). (**c**) iWAT ATP levels of the same animals. Data normalized to protein content (*n* = 4). (**d**) *Pyruvate dehydrogenase kinase 4* (Pdk4) and *phosphoenolpyruvate carboxykinase* (Pepck) mRNA levels in iWAT of the same animals (*n* = 4). (**e**) Immunoprecipitation (IP) of Ampk alpha 1 and blotting (IB) for Ampk beta 1 from iWAT and BAT of C26 and control animals. (**f**) Ampk alpha activity in aWAT (left), iWAT (middle), and BAT (right) of C26 cachectic animals (*n* = 6). (**g**) NEFA levels of Ampk alpha 1 overexpressing (OE) 3T3-L1 adipocytes treated with C26 CM (*n* = 6). (**h**) Immunoblot (representative for *n* = 4) of the lipolysis, lipogenesis and Ampk signaling pathways in iWAT of *Apc* delta580 heterozygous (*Apc*580/+)cachectic animals. (**i**) Immunoblot (*n* = 3) of nude BALB/c mice injected with human SW480 cachexia-inducing tumor cells. All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates n.s., non-significant. (**b,c,d,f**)Student’s t-test. (**g**) 2-way ANOVA with Bonferroni’s Multiple Comparison Posttest. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001

**Figure 3** WAT loss in cancer cachexia is not mediated by Ucp1.(**a**) Immunohistochemical staining of Ucp1 in iWAT of *Ucp1* knockout (KO) and wild-type (wt) mice injected with LLC tumor cells or PBS, sacrificed 21 d after tumor cell injection, and C26-injected wt animals. Ucp1 in cold-induced brite fat shown as comparison. Scale bars, 100 µm. (**b**) Change of body weight, body fat and lean mass in Ctrl, pair-fed (PF) and C26 mice housed at 30 °C measured by ECHO-MRI (Ctrl, PF *n* = 5, C26 *n* = 10). (**c**) Average tissue weights of gastrocnemius skeletal muscle (GC), inguinal white (iWAT), abdominal white (aWAT) and interscapular brown (BAT) adipose tissue depots of the same animals. (**d**) Serum NEFA levels of the same animals. (**e**) H&E staining of iWAT of the same animals. Scale bars, 20 µm. (**f**-**i**) Body weight (tumor weight subtracted) and tissue weights of *Ucp1* KO and wt mice injected with LLC tumors or PBS, sacrificed 21 d after tumor cell injection (LLC *n* = 5, PBS *n* = 8). All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates. (**b**-**d**,**f**-**i**) 1-way ANOVA with Tukey’s Multiple Comparison Posttest. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Figure 4** Cidea mediates disruption of Ampk integrity.(**a**) mRNA levels of *Ampk* alpha 1 and beta 1 in iWAT of C26 (left) and LLC (middle) cachectic animals (*n* = 6) and in 3T3-L1 adipocytes (right) treated with C26 conditioned medium (CM) (*n* = 4). (**b**) NEFA levels in 3T3-L1 supernatants of cells treated with control or *Cidea*-specific siRNA (*n* = 4). (**c**) Immunoblot (n=4-6) of iWAT from LLC-transplanted wild-type (wt) and *Cidea* knockout (KO) animals and quantification of Hsl-S565 versus total Hsl protein. (**d**)Relative NEFA levels in supernatants of iWAT (left) and aWAT (right) explants from wt and *Cidea* KO animals treated with control or C26 mouse serum (*n* = 3). All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates. n.s., non-significant. (**b,d**) 2-way ANOVA with Bonferroni’s Multiple Comparison Posttest.(**c**)Student’s t-test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Figure 5** CIDEA levels are elevated in human WAT under conditions of substantial weight loss. (**a**) Association between self-reported weight loss and *CIDEA* gene expression as obtained by microarray (*n* = 27). (**b**) Association between *CIDEA* gene expression and protein levels. Protein levels were quantified and normalized to ACTIN in samples from 22 individuals. (**c**) Relative *CIDEA* mRNA levels in subcutaneous (left) and omental (right) adipose tissue in weight stable (lean, *n* = 30) tumor-bearing patients and tumor-bearing patients with a pre-operative body weight loss of > 5% within a 3 months period (*n* = 33). (**d**)Relative *CIDEA* mRNA levels in intraabdominal omental fat depots from patients with obesity (14 women, 9 men) who underwent a two-step bariatric surgery strategy with gastric sleeve resection as the first step and a Roux-En-Y- gastric bypass as second step 12 ± 2 months later. (**e**) Association of *CIDEA* expression fold change and change in serum free fatty acids (FFAs) of the patients that underwent bariatric surgery. (**f**) Flag IP and blot for Cidea in primary adipocytes differentiated in control or C26 mouse serum. (**g**) V5 IP and blot for Ampk beta 1 in wt or Ampk beta KO MEFs transfected with V5-Cidea and control peptide (CP) or Ampk-Cidea interfering peptide (ACIP). (**h**) IP of Ampk alpha 1 and blot for Ampk beta 1 in primary adipocytes differentiated in control or C26 mouse serum and transfected with CP or ACIP. (**i**) Immunoblots (representative of *n* = 4) from primary adipocytes differentiated in control and C26 mouse serum, transfected with control peptide (CP) or inhibitory peptide (ACIP).(**j**)NEFA and glycerol levels in supernatants of primary adipocytes differentiated in either control or C26 mouse serum, with CP or ACIP (*n* = 4). All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates. (**a,b,e**)Pearson correlation. Associations were evaluated using linear regression. *R*- and *P*-values are indicated. (**c**,**d**) Student’s t-test. (**j**) 2-way ANOVA with Bonferroni’s Multiple Comparison Posttest. \**P* < 0.05, \*\*\**P* < 0.001.

**Figure 6** ACIP reduces WAT wasting in cachexia. (**a**)Flag-ACIP expressing adeno-associated virus. ITR (inverted terminal repeats), *pAdipoq* (Adiponectin promoter), *miR122* binding site), *SV40pA* (simian virus poly A). (**b**) iWAT weights of PBS (*n* = 6) or C26 cell injected (*n* = 10) mice, injected with Ctrl (C) or flag-ACIP AAV into the left or right inguinal fat pad. (**c**) H&E staining of iWAT of the same C26 animals. Representative of 3 different mice. Scale bars, 20 µm. (**d**) Immunoblots (*n* = 3) of the Ampk signaling pathway from iWAT of the same animals. (**e**) Body weight, fat mass and lean mass change of control (PBS *n* = 4) or cachectic (C26 *n* = 12) animals injected with AAV without or with flag-ACIP (C, ACIP) or with NaCl (Ctrl). (**f**) Weights of iWAT (left) and aWAT (right) of the same animals. (**g**) IP of Ampk alpha 1 and blot for Ampk beta 1 in iWAT of PBS or C26 cell injected animals and C or ACIP AAV. (**h**) Immunoblot (*n* = 3) of Ampk phosphorylation targets from iWAT of the same animals. (**i**) Percentage of cachexia-free mice in C or ACIP AAV injected animals with C26 tumors as in **e**-**h**. (**j**) Percent body weight change and inguinal and abdominal fat pad weights of control (PBS *n* = 4) or cachectic (SW480 *n* = 8) SCID mice injected intravenously with ACIP or Control (C) AAV prior to SW480 tumor cell injection, sacrificed 16 d later. All data in the figure are shown as the mean ± s.e.m. *n* numbers refer to biological replicates. (**b,j**) Student’s t-test. (**e-f**) 2-way ANOVA with Bonferroni’s Multiple Comparison Posttest. \**P* < 0.05.

**ON-LINE METHODS**

**Animal experiments.** Eight to ten week-old male BALB/c, C57Bl6/N, Fox Chase SCID and nude BALB/c mice were obtained from Charles River Laboratories (CRL, Brussels). All mice were maintained on a 12 h light-dark cycle at 22 °C with regular unrestricted diet (Kliba Nafag #3437, Provimi Kliba AG, Kaiseraugust) unless stated otherwise. Mice were injected with 1.5\*106 C26 colon carcinoma cells, 2\*106 LLC cells, and 5\*106 SW480 cells, respectively, subcutaneously into the right flank. Tumor growth, body weight and body composition were measured for ~20 d post tumor cell injection. For thermoneutrality experiment, mice were housed at 30 °C two weeks prior to injection and kept at 30 °C during the whole experiment. Food was weighed daily and a pair-feeding group was established where indicated when C26 mice displayed symptoms of anorexia (at about 13-18 d after the C26 cell injection). The pair-fed mice were then paired to a cachectic mouse such that the pair-fed mouse was given the same amount of food, by weight, as was consumed by the tumor bearing mouse on the previous day.

Mice were killed at 9-11 a.m. by cervical dislocation when they had lost 10-15% body weight or at the latest 24 d after the tumor cell injection. Organs including tumor, liver, fat pads (inguinal WAT, iWAT; epididymal/abdominal WAT, aWAT; and interscapular BAT), and gastrocnemius muscles (GC) were collected, snap frozen and used for further analysis. Total body fat content was determined by an Echo magnetic resonance imaging (ECHO-MRI) body composition analyzer (Echo Medical Systems, Houston). Indirect calorimetry was performed in PhenoMaster cages (TSE Systems, Bad Homburg) with individually housed mice at 22 °C; measurements were performed and statistically analyzed as described[39](#_ENREF_39). For the pair-fed group, food access was blocked when the control mice had consumed the amount of food that the C26 mice had consumed the hour before. C26 injected animals were sacrificed when they had lost > 10% body weight; the respective pair-fed control was sacrificed on the same day.

6-hydroxydopamine (6-OHDA, Sigma, Munich) was dissolved in saline solution (Braun, Melsungen) with 1% ascorbic acid (Sigma, Munich). While under anesthesia of isofluorane, mice underwent surgery where each iWAT depot was injected 14 times at different sites (2 µl per injection) with 10 mg/ml 6-OHDA or saline solution. The mice were allowed to recover for a minimum of 10 d prior to C26 or PBS injection. For the cold exposure experiment, mice were left to recover for 10 d before being placed in the TSE Phenomaster for 24 h at 22 °C followed by 24 h at 4 °C. Tissue norepinephrine levels were determined using commercially available ELISA kits (Abnova, Heidelberg).

*Apc* delta580 mice were obtained from NCI Frederick and housed until heterozygous animals developed multiple intestinal neoplasia and cachexia at five to six months of age[38](#_ENREF_38). *Ucp1* and *Cidea* knockout mice have been described before [15](#_ENREF_15),[41](#_ENREF_41).

In each animal experiment, mice were randomly assigned to each group. Animal handling and experimentation was performed in accordance with the European Union directives and the German animal welfare act and approved by the Regierungspräsidium Karlsruhe, Germany.

**Recombinant viruses.** Ampk beta 1 amino acids 232-248 and n-terminal Flag tag were cloned into puc57-pAdiponectin-miR122. Recombinant adeno-associated viruses (AAV) were produced by co-transfection of HEK 293 cells with puc57 and pDP8.ape plasmids (Plasmid factory, Bielefeld) and purified by VectorBiolabs (Philadelphia, PA). Control AAV represents an empty virus without peptide. Mice were injected at 3\*109 ifu per fat pad by microinjection into the inguinal fat pad (12 injections at different sites with 4 µl each) as described above. In a separate experiment, mice were injected with 5\*1011 ifu into the tail vein. Experiments were initiated two to three weeks following virus infection.

Mature 3T3-L1 adipocytes were transduced with a constitutively active Ampk alpha adenovirus[56](#_ENREF_56) and a GFP adenovirus as control at an MOI 500, respectively. After three days, cells were treated with C26 CM for 24 hrs and lipolysis was measured.

**Histology.** Tissues were collected and immediately stored in 4% paraformaldehyde for 24 h at 4 °C. Paraffin embedded tissues were cut in 4 μm sections and mounted. Sections were stained with hematoxylin and eosin by standard procedures. Immunohistochemistry was performed using the α-Flag antibody (F1804, 1:1,000, Sigma, Munich) and Ucp1 antibody (ab23841, 1:1,000, Abcam, Cambridge). Stainings were imaged at 40x magnification (Zeiss, Jena).

**Quantitative Taqman RT-PCR**. Total RNA was extracted from frozen organ samples or cultured adipocytes using QIAzol and the RNeasy kit (Qiagen, Hilden). cDNA was prepared by reverse transcription using M-MuLV enzyme and Oligo dT primer (Fermentas, St. Leon-Rot). cDNAs were amplified using assay-on-demand kits and a StepOne Real-time PCR system (Life Technologies, Darmstadt). RNA expression data were quantified according to the delta Ct method as described[57](#_ENREF_57) and normalized to levels of *TATA-box binding protein* RNA (*Tbp*).

**Primary adipocytes isolation and differentiation.** Six to seven week old male NMRI mice were obtained from Charles River (Brussels, Belgium). Inguinal WAT and interscapular BAT were dissected and stromal vascular fractions were isolated as previously described[40](#_ENREF_40). Primary adipocytes were differentiated in standard differentiation cocktails[58](#_ENREF_58) unless otherwise stated. Serum of either control or cachectic mice from completed experiments was pooled and added to the differentiation cocktail at a concentration of 1% for both the differentiation induction and the differentiation maturation. Fetal Bovine Serum (FBS; Gibco, Darmstadt) was added at a concentration of 9% or 4% for induction and maturation, respectively. Media was replaced every other day. For indicated experiments, primary adipocytes were transfected with control Flag peptide (CP) or Flag-Ampk-Cidea Interfering Peptide (ACIP), 48 h post-isolation and 48 h prior to the induction of differentiation using Turbofect transfection reagent (Thermo Scientific, Schwerte) as per the manufacturer’s instructions.

**Cell culture.** Wild-type mouse embryonic fibroblasts (MEFs) or *Ampk* beta1beta2 KO MEFs[32](#_ENREF_32) were cultured in 5 g/l DMEM (Gibco, Darmstadt), 10% FCS and 1% Pen/Strep. Cells were transfected (Turbofect; Thermo Scientific, Schwerte) with V5-tagged Cidea overexpression construct with or without CP or ACIP for 48 h. Cells were lysed and analyzed by co-immunoprecipitation for interaction between Cidea and Ampk beta 1. 3T3-L1 cells (ATCC, Manassas, VA) were cultured and differentiated as described before[59](#_ENREF_59). Differentiated 3T3-L1 adipocytes were trypsinized, re-plated on 12 well plates and transfected with 60 nM siRNA (si*Cidea* #SI00168483 and negative control siNC #SI03650325; Qiagen, Hilden) using Lipofectamine2000 (Thermo Scientific, Schwerte) following manufacturer’s instructions. CP and ACIP: oligos coding for Ampk beta 1 amino acids 232-248 (AA sequence: Asn His Val Met Leu Asn His Leu Tyr Ala Leu Ser Ile Lys Asp Gly Val) were cloned into pLKO-puro FLAG (Addgene, Cambridge, MA). Control peptide was pLKO-puro HA-FLAG. For tumor cell conditioned media experiments, C26[26](#_ENREF_26), MC38, SW480, LLC and HT29 cells (ATCC, Manassas, VA) were plated in 15 cm plates, grown to 80% confluency, and the media were collected 48 h later. Media from 15 cm plates with no cells was used as control media. Media was filtered and was used 3:1 with fresh media for 24 h stimulation. Cell lines were regularly tested for mycoplasma contamination as required by in-house policies. Every cell culture experiment was repeated at least 2-3 times with *n* ≥ 3 technical replicates each time.

**Lipolysis and lipogenesis.** Serum was taken from animals immediately following sacrifice. Non-esterified free fatty acids (NEFA) and glycerol were quantified using 4 µl serum in commercial kits (Wako, Neuss; Sigma, Munich). For the lipolysis assay, 3T3-L1 and primary adipocytes were stimulated as described[59](#_ENREF_59) and NEFA and glycerol were quantified following 3 h of incubation and normalized to protein concentration by BCA assay (Thermo Scientific, Schwerte) where indicated. AICAR (final conc. 100 µM; Sigma, Munich) or H89 dihydrochloride (final conc. 50 µM; Merck Millipore, Darmstadt) were added during the 3 h incubation. For the lipogenesis assay, 3T3-L1 and primary adipocytes were stimulated with tumor cell conditioned media for 24 h, then washed twice with 1 x PBS and incubated with Krebs-Ringer buffer for 1 h. Lipogenesis was measured in cells stimulated with Krebs-Ringer buffer containing 0.5% BSA, 25 mM HEPES and 5 mM glucose, spiked with 5 µl Glucose, D-[14C(U)] (Perkin Elmer, Baesweiler, Germany) per well for 2 h. Cells were washed twice and harvested in 0.5 M NaOH. Lipids were extracted by chloroform/methanol extraction, and glucose incorporation into lipids was measured by scintillation counting.

**Adipose tissue explants**. 14 month old female *Cidea* knockout mice and three month old female C57BL6 mice from in-house breeding colonies (DKFZ, Heidelberg) were used to isolate abdominal and inguinal WAT. Tissue was transferred to ice cold 1 x PBS and lymph nodes and larger blood vessels were removed. Tissues were cut into ~2 mm sized pieces. Pieces were transferred to 24 well plated containing pre-warmed culture medium (DMEM high glucose and 10% FBS). Once all tissues were collected medium was exchanged to culture medium containing 2% mouse serum from C26-bearing mice or control animals and 8% FBS. During incubation culture plates were kept on an orbital shaker. After 24 h lipolysis assay was started. Explants were collected in N2 and weighed for normalization.

**Triglyceride isolation and determination.** Differentiated 3T3-L1 adipocytes were trypsinized and re-plated on 6 well plates. One day after seeding, the cells were treated with tumor cell conditioned medium for 24 h. Cells were harvested in 200 µl of Triton™ X-100 (TX) lysis buffer (150 mM NaCl, 0.05% Triton™ X-100, 10 mM Tris/HCl (pH 8.0), 1x Protease Inhibitor Cocktail), frozen at -80 °C for at least 24 h and sonicated after thawing for two cycles (one cycle: 30 sec on and 30 sec off). Cell debris was removed by centrifugation. Protein concentration was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Schwerte). Triglyceride (TG) levels were determined using the Serum TG Determination Kit (Sigma Aldrich, #TR0100). TG content (TG-bound glycerol) was determined by subtracting the free glycerol (blank value) from the total glycerol (assay value).

**Extracellular flux analysis.** 4,000 3T3-L1 cells per well were seeded onto an XF96 PS microplate (Seahorse Bioscience, Copenhagen, Denmark) which was coated with 0.2% gelatin. After cells reached confluency they were differentiated as described before[59](#_ENREF_59). Differentiated 3T3-L1 cells were exposed to tumor cell conditioned medium 24 h prior to the analysis. Mitochondrial Stress Test was performed according to manufacturer´s instructions (Seahorse Bioscience, Copenhagen, Denmark). Assay compounds were used at the following final concentrations: 4 µM oligomycin, 0.75 µM FCCP, 0.5 µM rotenone and 0.5 µM antimycin A. Mitochondrial function assessed by oxygen consumption rate (OCR) was normalized to cell density (absorbance) determined by Sulforhodamine B staining.

**Protein analysis.** Proteins were extracted from frozen organ samples or cultured adipocytes following lysis in ice cold lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% Triton X, 1 mM EDTA, 1 mM Na3V04, 1 mM NaF, 1 µg/ml pepstatin A and 1 mM DTT) and extracts were separated on 10-15% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blot assays were performed using antibodies specific for Actin (A5441, Sigma, Munich; 1:5,000), Acc-S79 (#3661; 1:3,000), Acc total (#3662; 1:3,000), Hsl-S565 (#4137), Hsl-S660 (#4126), Hsl total (#4107), Ampk alpha (#2532; 1:2,000), Ampk alpha-T172(#2531), Ampk beta 1-S108 (#4181), phosphorylated Pka targets (#9624), Atgl (#4126) (all Cell Signaling, Danvers), Ampk beta 1 (ab32112; 1:5,000), Perilipin (ab3526), V5 (ab15828), VCP (ab11433; 1:10,000) (all Abcam, Cambridge), Cidea (NBP1-76950 Novus Biologicals, Cambridge; 1:500). Antibodies were used in a 1:1,000 dilution unless stated otherwise. Validation data are provided on the manufacturer’s websites, respectively.

For immunoprecipitation, 500 µg protein was pre-washed in 40 µl protein A/G agarose (sc-2003, Santa Cruz, Heidelberg) and 400 µl lysis buffer (see above) for 1 h at 4 °C. After spinning (13,000 rpm, 4 °C, 1 min) supernatants were collected and incubated with 4 µg of the respective antibody over night at 4 °C on a rotating wheel. The next day, 40 µl of protein A/G agarose was added to the samples and rotated for additional 2 h. Protein-bound beads were washed five times with lysis buffer and proteins were eluted by the addition of SDS sample buffer and subjected to immunoblotting.

**ATP assay.** ATP levels were quantified from 3T3-L1 adipocytes harvested in 100 µl ATP assay buffer or from 20-30 mg iWAT tissue harvested in 200 µl assay buffer and lysed with the tissue lyser following the manufacturer’s instructions (ATP Assay Kit, Abcam, Cambridge).

**Ampk activity assay.** Samples of adipose tissue (BAT, aWAT, iWAT) were collected by using freeze-clamping with liquid nitrogen. Frozen tissues were crushed and mixed with lysis buffer (50 mM Tris, pH 7.4; 1 mM EDTA; 0.15 M NaCl; 1 mM benzamidine; 1 mM dithiothreitol; 50 mM sodium fluoride; 5 mM pyrophosphate tetrasodium; 1 mM phenylmethylsulfonylfluoride; 0.2% Triton X-100; 1% glycerol; 10 mg/ml aprotonin; 10 mg/ml pepstatin; 10 mg/ml leupeptin and Phosphatase Inhibitor-Mix I, (Serva, Heidelberg, Germany) under liquid nitrogen. Tissue lysates were sonicated and centrifuged at 18,000 g for 10 min at 4 °C. Protein concentration in the supernatants was measured by BCA assay (Thermo Scientific, Schwerte, Germany).

Activities of alpha 1 and alpha 2 Ampk isoforms were measured as before[60](#_ENREF_60) using a peptide substrate[61](#_ENREF_61). Briefly, Ampk alpha 1 and 2 isoforms were separately immunoprecipitated from the tissue extracts by using specific sheep antibodies bound to Protein G Sepharose (GE Healthcare Bio-Sciences AB, Sweden). The activity of precipitated protein was determined in a HEPES-Brij buffer containing AMP (1 mM), [γ-32P]ATP (1 mM) and AMARA peptide (1 mM; Vidia Prague, Czech Republic). After 15 min at 30 °C, the reaction was stopped by washing with 1% phosphoric acid solution using paper filters. The activity of 1 U/mg protein corresponds to 1 nmol 32P-AMARA peptide/mg protein per min.

**Patient cachexia studies**

27 patients scheduled for gastrointestinal cancer operation were investigated. The patients have been examined before and clinical data are reported[30](#_ENREF_30),[62](#_ENREF_62). Prior to surgery, they came to the clinical research unit after an overnight fast for relevant clinical examinations and a needle biopsy from adipose tissue. They had not received any anti-cancer treatment and did not have clinical evidence of gastrointestinal obstruction or jaundice. The primary cancer location was pancreas (*n* = 17), stomach (*n* = 2), colon (*n* = 4), oesophagus (*n* = 1), gall bladder (*n* = 1), and liver (*n* = 2). Among the collected clinical parameters, self-reported unintentional weight loss in the three to six months prior to the investigation was used for comparisons with gene and protein expression. The study was approved by the Karolinska (Stockholm, Sweden) ethics committee. The investigation was explained in detail to each patient and written informed consent was obtained.

*Fat biopsies*

The procedures have been described in detail[62](#_ENREF_62). After clinical examination, an abdominal subcutaneous fat sample was obtained by fine needle biopsy. Tissue pieces were rapidly rinsed in saline and aliquots (about 300 mg each) were frozen in liquid nitrogen and kept at -70 oC for subsequent global gene expression profiling.

*Analysis of protein expression*

150 ug of total protein were separated by 8-16% gradient SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and Western blot was performed according to standard procedures. The membranes were blocked in 3% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, UK). Primary antibodies against CIDEA (# C7987, 1:1,000; Sigma-Aldrich, St. Louis, MO) were used. ACTIN (# A2066, 1:1,000; Sigma-Aldrich, St. Louis, MO) was used as a loading control. Images were analyzed with Quantity One Software (Bio-Rad Laboratories), and values were expressed as fold change relative to the weight stable subjects.

*Gene expression*

We used data from WAT global gene expression profiling which has been published before[62](#_ENREF_62) and data have been uploaded to GEO (GSE20571). In brief, RNA was prepared and biotinylated complementary RNA was hybridized to Affymetrix Gene 1.0 ST Arrays using standardized protocols (Affymetrix Inc., Santa Clara, CA, USA). Subsequent data analyses were performed using the Affymetrix GeneChip Operating Software (GCOS) version 1.4. To allow comparisons of transcript levels between samples, all samples were subjected to an all-probe set scaling-to-target signal of 100. In the present study, we only studied the expression of *CIDEA*.

**Human bariatric surgery intervention and cachexia study**

We included 23 Caucasian obese subjects (14 women, nine men) who underwent a two-step bariatric surgery strategy with gastric sleeve resection as the first step and a Roux-en-Y- gastric bypass as second step 12 ± 2 months later. In addition, we included adipose tissue samples from a cross-sectional cohort of 63 individuals with leanness, but stable body weight three months prior to surgery (BMI: 20.63 ± 1.8 kg/m²; *n* = 30) or tumour associated cachexia defined as weight loss > 5% in the two months prior to surgery (BMI: 20 ± 2.1 kg/m²; *n* = 33). Patient details are displayed below:

|  |  |  |
| --- | --- | --- |
| **Parameter** | **Cachexia** | **Weight stability** |
| *n* (Women/Men) | 33 (19/14) | 30 (16/14) |
| Cancer type (*n*):  Rectal  Sigma  Colon  Gastric  Cholecystic  Bladder | 7  6  17  0  2  1 | 7  4  16  1  2  0 |
| Age (years) | 62 ± 14 | 59 ± 18 |
| BMI (kg/m²) | 20.1 ± 2 | 20.6 ± 1.8 |
| Creatinine (µmol/l) | 74 ± 31 | 90 ± 75 |
| C-reactive protein (mg/l) | 21 ± 41 | 21 ± 27 |
| Chemotherapy | none | none |

Serum or plasma samples, omental and subcutaneous adipose tissue were obtained. General inclusion and exclusion criteria as well as methods for the measurement of anthropometric and laboratory parameters have been reported recently[63](#_ENREF_63). Study protocols have been approved by the ethics committee of the University of Leipzig (Reg. No. 031-2006 and 017-12-23012012). All participants gave written informed consent before taking part in the study.

*Characterization of human adipose tissue samples*

Adipose tissue samples were taken from the abdominal subcutaneous and the intraabdominal omental fat depots at defined locations during surgery. Adipose tissue was analyzed as a whole (immediately frozen in liquid nitrogen after explantation) for histology and mRNA expression analyses. *CIDEA* mRNA expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler using TaqMan assay-on-demand kits (Hs00154455\_m1; Applied Biosystems), and fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). *CIDEA* mRNA expression was calculated relative to the mRNA expression of *Hypoxanthin-Guanin-Phosphoribosyltransferase 1* (*HPRT1*) (Hs01003267\_m1; Applied Biosystems).

**Statistical analysis.** For each experiment, means and s.e.m. of parameters measured were determined. Statistical analyses were performed using Student’s t-test in one-factorial designs. For multi-factorial study designs, one- or two-way ANOVA were used as appropriate. Tukey’s or Bonferroni post hoc was applied, respectively, when significant differences were found. Testing for normal variance was performed and confirmed. For animal experiments, number of animals per group to detect biologically significant effect sizes was calculated using appropriate statistical sample size formula and indicated in the biometrical planning section of the animal license submitted to the governing authority. Blinding was not done during animal group allocation but in some measurements made in the study (*i.e.*, Histology, AMPK activity assay). No specific exclusion criteria were applied as inbred strains were used exclusively that display uniform phenotypic characteristics. Analyses were carried out with SigmaPlot v.12 software (Systat Software GmbH, Erkrath) or GraphPad Prism software (GraphPad Software, San Diego). *P* < 0.05 was considered statistically significant.

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